AWARD NUMBER: W81XWH-10-2-0121

TITLE: Hibernation-Based Therapy to Improve Survival of Severe Blood Loss

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REPORT DATE: June 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2016	Final	1 October 2010 to 31 March 2016

4. TITLE AND SUBTITLE
Hibernation-Based Therapy to Improve Survival of Severe Blood Loss

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-2-0121

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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200 Oak Street S.E., Suite 450
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have provided data that provide evidence that BHB/M is a safe and effective therapy when given at the proper dose and concentration can decrease mortality and improve outcomes for injured casualties suffering from polytrauma and blast injuries.

15. SUBJECT TERMS
Beta-hydroxybutyrate, melatonin, shock, poly-trauma

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	38	

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Introduction:

Blast injuries have been responsible for the majority of combat deaths in Iraq and Afghanistan, and the likelihood of being exposed to explosives is increasing for military personnel and civilians alike in war zones and other regions of political conflict. The injuries sustained are often accompanied by severe blood loss, and shock from this blood loss is the most common cause of potentially salvageable deaths from combat related injuries. D-beta hydroxybutyrate and melatonin (BHB/M) is a novel therapy designed to prolong survival in patients who are risk for bleeding to death. Our overall strategy in this series of studies is to use physiologic adaptive responses in hibernating mammals to aid in salvage of a patient with a potentially life-threatening blood loss, permitting survival to reach effective medical care. BHB/ M includes both an alternate fuel source for cells (D-beta hydroxybutyrate) and a powerful anti-oxidant, melatonin, to protect cells against damage. Our goal is to evaluate BHB/M in animal models of injury that simulate the battlefield casualty. Our previous work has shown increased survival for both rats and pigs treated with BHB/M. We wish to prove that BHB/M is a safe and effective therapy that can decrease mortality and improve outcomes for injured casualties suffering from polytrauma and blast injuries.

Key Words:

Beta-hydroxybutyrate

Melatonin

Poly-trauma

Resuscitation

Hemorrhage

Shock

Specific Aim 1. Toxicity and Maximu Tolerated MTD of BHB/M

Deliverable Aim 1a: Identification of No Observable Adverse Effect Level (NOAEL)
Assess 3 dosing levels and two control groups to address the dose response relationship for the toxicity of the drug product via I.V. infusion in a rabbit ear vein (6 groups; 2 ears/group , n=24 ears. Two sacrifice time points (24 hours and 72 hours), n=12 ears at 24 hours and 12 ears at 72 hours.

Previous studies had shown a local irritation of the vein and tissue at the site of injection of BHB/M (1). The aim of our rabbit study was to establish the non-observable adverse event level (NOAEL) of BHB/M at the injection site when administered intravenously. We wished to assess four dosing levels along with two control groups to address the dose response relationship for the toxicity of the drug product utilizing intravenous (IV) infusion in a rabbit ear vein as well as establish NOAEL (Table 1).

Table 1. Experimental Design for Task 1

Randomization	Euthanasia 24 hours	Euthanasia 72 hours
Normal Saline	2	2
20% DMSO	2	2
BHB/M 1:10	2	2
BHB/M 1:5	2	2
BHB/M 1:2	2	2
BHB/M 1:1	2	2

Rabbit ear veins were infused with normal saline, 20% DMSO, and 2M test solution or 4M test solution (both test solutions with DMSO vehicle). At 24 or 48 hours post administration the rabbit ears were fixed in 10% formalin.

Findings:

We identified NOAEL with the following groups of animals (Table 2). No grossly identified adverse events or venous thrombosis were noted in any of the groups. Tissues were fixed in 10% formalin and processed using H&E staining.

Table 2. Completed Randomization Groups

Randomization	Euthanasia 24 hours	Euthanasia 72 hours
Normal Saline	2	2
20% DMSO	2	2
BHB/M 1:2	2	2
BHB/M 4M	2	2

Samples were evaluated with histology, read by a blinded, licensed veterinary pathologist utilizing the grading scale in Table 3.

Table 3. Histological Grading Scale

Grade	Scale
1	Minimal
2	Mild
3	Moderate
4	Marked

Summary of histopathology findings compared by group

Saline versus DMSO injections

- Saline injections, in general, were associated with relatively benign lesions, e.g., perivascular hemorrhage
- DMSO may cause more significant lesions (vascular damage and thrombosis, dermal leukocyte infiltrates etc.), presumably if it leaks extravascularly
- Necrosis and inflammation involving the ear tip is considered to be a more severe manifestation of vascular damage associated with the injection
- Lesions with DMSO appear to be somewhat less severe at 72 hours

2M test solutions versus DMSO injections

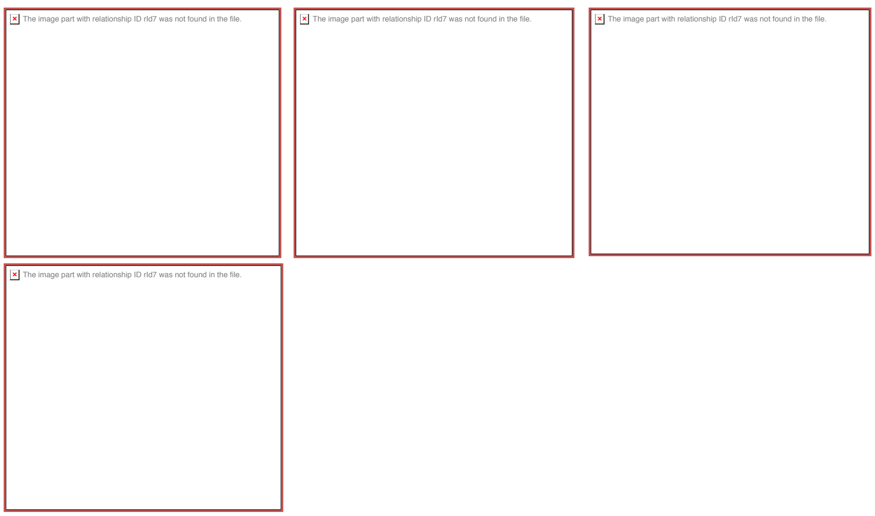
- Although DMSO induced similar lesions to the 2M test solution, it appears that 2M test solution is more likely to cause vascular necrosis and inflammation (noted at 24 hours).

- Necrosis and inflammation involving the ear tip was observed at a higher incidence with the 2M solution.

4M test solutions versus DMSO injections

- Although DMSO induced similar lesions to the 4M test solution, it appears that 4M test solution is more likely to cause vascular necrosis and inflammation (noted at 24 and 72 hours)(Figure 1).

Figure 1. Gross appearance of rabbit ears with no obvious necrosis at 24h or 72h



Comment:

Microscopic findings were observed with all solutions injected. These were considered to be more severe (e.g., vascular damage and dermal inflammation) with DMSO than the benign lesions (e.g., perivascular hemorrhage) observed with normal saline, but were not present in all samples suggesting that more severe lesions may be associated with vascular leakage of the DMSO. There appears to be a tendency for slightly greater vascular damage with the 2M and 4M test solutions than with DMSO, however a study with larger numbers would be needed to confirm that this is indeed the case. The presence of necrosis of the ear tips in a number of animals treated with DMSO vehicle, 2M test solution or 4M test solution is considered to be a reflection of more severe vascular damage (e.g., vascular necrosis and thrombosis in the affected animals. In

general, lesions appear to have ameliorated at 72 as opposed to 24 hours post administration.

With the pathology supporting our decision to end further rabbit ear vein studies, we proceeded with our pig studies using 4M BHB/M (pH 7.4) for our infusion treatment.

Conclusion:

A pH-neutral mixture of 4M-d-beta Hydroxybutyrate and 43mM-Melatonin solution is not associated with long-term severe vascular or tissue necrosis and is safe to administer via peripheral vein.

Deliverable Aim 1b: Determination of Maximum Tolerated Dose (MTD) of BHB/M

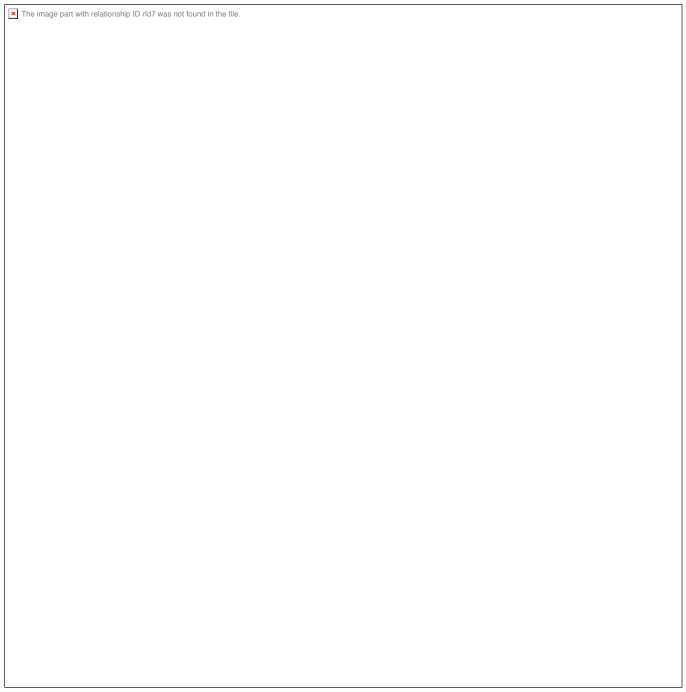
What is the Maximum Tolerated Dose (MTD) of 4M BHB/43mM Melatonin in our model of shock/polytrauma? Two study groups (I.V. 4M BHB/43mM Melatonin, 2X (3 Male, 3 Female) or I.V. 4M BHB/43mM Melatonin, 4X (3 Male, 3 Female)), n=12 are to be randomized utilizing the below experimental grid (Table 4).

Table 4.

Drug Components	Concentration of Drug	Dose	Number of Animals
I.V. BHB/M 2X	4M BHB/43mM Melatonin	2 cc/kg bolus 1.32cc/kg/hr	3 Male, 3 Female
I.V. BHB/M 4X	4M BHB/43mM Melatonin	4 cc/kg bolus 2.64 cc/kg/hr	2 Male, 3 Female

All animals (n=5) receiving 4X BHB/M had died about 5 hours after the start of BHB/M infusion. Two of the animals receiving 2X BHB/M (n=6) died within the first 10 hours BHB/M infusion, two of the animals died as they were unable to be extubated and 2 of the animals survived until end of experiment (Figure 2).

Figure 2. Kaplan Meier Curve for observation of Maximum Tolerated Dose (MTD)



Unsurprisingly, BHB concentrations are significantly different ($p<0.05$) in those animals treated with 2X BHB/M at LR 1 and FR 2 when compared to animals treated with 4X BHB/M at the same time points (Figure 3). The same effect is seen in Melatonin concentrations (Figure 4).

Figure 3. BHB concentrations in animals treated with either 2X or 4X BHB/M

Figure 4. Melatonin concentrations in animals treated with either 2X or 4X BHB/M

Both wedge and bladder pressures were significantly different in animals receiving 4X BHB/M when compared to those receiving 2X BHB/M ($p<0.05$) (Figure 5 a and b). Arterial pH, lactate and sodium concentrations were significantly different between the groups ($p<0.05$) (Figure 6 a, b and c). All of these measures, both physiologic and hematologic would indicate that there is an issue with fluid flow that is dependent upon the dosing of BHB/M.

Figure 5 a) Wedge pressure and **b)** bladder pressure in animals treated with either 2X or 4X BHB/M.

a.

b.

Figure 6 a) Arterial pH **b)** serum lactate and **c)** serum sodium concentrations in animals treated with either 2X or 4X BHB/M.

a.

b.

c.

Pathology reports noted some kidney necrosis in a few animals treated with either 2X or 4X 4 M BHB/43mM Melatonin. All other pathologic abnormalities were as a result of the poly-trauma.

Deliverable Aim 1c: Toxicity profile of BHB/M

Maximum tolerated dose (MTD) of the individual components of BHB/M were tested (Table 5). As it became evident that increased doses (4X) of the individual components were not associated with improved outcome, that set of experiments stopped and additional studies, with no injury, were added to determine whether the animals were dying from injury, drug or a combination of both (Table 5).

Table 5. Summary of MTD with Injury and MTD with No Injury Experiments.

Drug Components	Concentration of Drug	Animal Sex
I.V. BHB 4X	4M BHB	Male
I.V. Melatonin 4X	43mM Melatonin	Female
I.V. DMSO 4X	20% DMSO	Male
Lactated Ringers' 4X	Lactated Ringers'	Female
I.V. BHB 4X	4M BHB	Male
I.V. DMSO 4X	20% DMSO	Female
I.V. BHB 4X	4M BHB	Female
I.V. Melatonin 4X	43mM Melatonin	Female
I.V. DMSO 4X	20% DMSO	Female
I.V. BHB 4X No Injury	4M BHB	Male
I.V. DMSO 4X No Injury	43mM Melatonin	Male
I.V. Melatonin 2X No Injury	43mM Melatonin	Female
I.V. BHB 2X No Injury	4M BHB	Male
I.V. Melatonin 4X No Injury	43mM Melatonin	Female
I.V. BHB 2X No Injury	4M BHB	Female
I.V. Melatonin 2X No Injury	43mM Melatonin	Male
I.V. BHB 4X No Injury	4M BHB	Female

3 animals received injury and 4x 4M BHB

- 1 died after Shock 35, 2 died just prior to FR 7

2 animals received injury and 4x 43mM Melatonin

- 1 survived until FR 7, 1 died after Shock 35

1 animal received injury and 4x Lactated Ringers'

- 1 survived until FR 7

3 animals received injury and 4x 20% DMSO

- 1 animal died after LR 1 and 2 animals died after Shock 35

2 animals received no injury 2x 4M BHB

- 2 animals survived until FR 7

2 animals received no injury 2x 43mM Melatonin

- 2 animals survived until FR 7

2 animals received no injury 4x 4M BHB

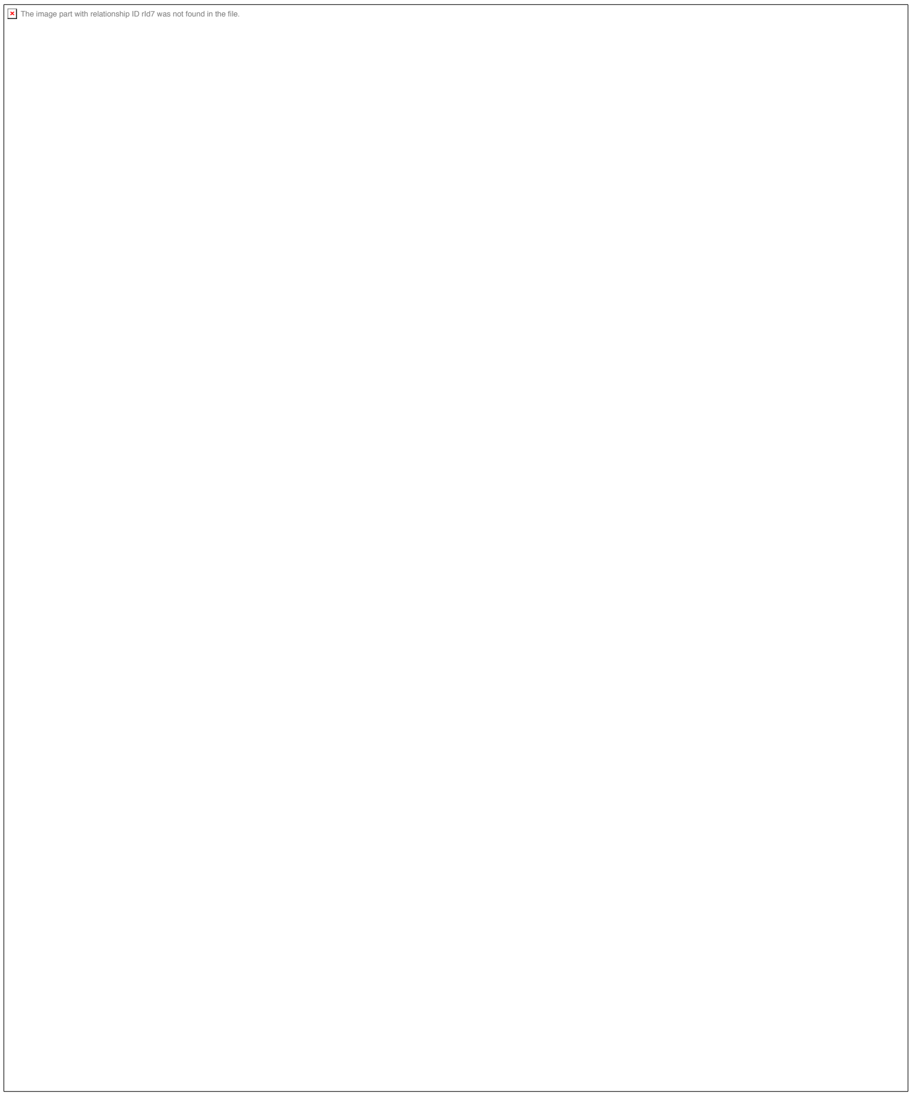
- 2 animals survived until FR 7

2 animals received no injury 4x 43mM Melatonin

- 2 animals survived until FR 7

The animals that had injury and received a 4x dose of 4M BHB had consistently high serum concentrations from Shock 35 and started to decrease after FR 2 (roughly when the BHB infusion is complete). Animals that had no injury and a 4x dose of 4M BHB had increasing serum concentrations of BHB which peaked at FR 2 and decreased until levels returned to near Baseline at FR 7. Animals having no injury and receiving a 2x dose of 4M BHB had fairly consistent serum concentrations of BHB until FR 2 at which time they started to decrease reaching near Baseline levels at FR 7 (Figure 7).

Figure 7. BHB concentrations in animals receiving injury and no injury at various dosing concentrations



The animals that received both injury and a 4x dose of 43mM Melatonin exhibited higher serum concentrations of melatonin. The concentrations of melatonin peaked at Shock 35 and continued to go down throughout the experiment. Animals receiving 4x dose of 43mM Melatonin and had no injury exhibited increasing serum concentrations of melatonin that peaked at FR 2 and continued to decrease until the end of the experiment. Animals that had no injury and received a 2x dose of 43mM Melatonin had consistent levels of melatonin that decreased after FR 190 (Figure 8).

Figure 8. Melatonin concentrations in animals receiving injury and no injury at various dosing concentrations

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Specific Aim 2. Toxicity of Individual Components of BHB/M at MTD

Deliverable Aim 2: Evaluation of the contribution o the individual components of BHB/M to the toxicity profile of the product at MTD.

Observation of the preservation of survival benefit using doses of melatonin at various concentrations that have not been previously studied in conjunction with 4M BHB. Six study groups (Lactated Ringers' (3 Male, 1 Female), 4M BHB/43mM Melatonin (3 Male, 3 Female), 4M BHB/20mM Melatonin (3 Male, 3 Female), 4M BHB/10mM Melatonin (3 Male, 3 Female), 4M BHB/4.3mM Melatonin (3 Male, 3 Female), 4M BHB/0.43mM Melatonin (3 Male, 3 Female)), n=32. The following randomization grid was utilized (Table 6).

Table 6.

Drug Component	Concentration of Drug component	# of Animals Male	# of Animals Female
Lactated Ringers'	10 cc/kg, 0.66 cc/kg/hr	3	1
BHB/M I.V.	4 M BHB/43 mM melatonin, 20% DMSO	1	1
BHB/M I.V.	4 M BHB/20 mM melatonin, 5% DMSO	3	3
BHB/M I.V.	4 M BHB/10 mM melatonin, 5% DMSO	3	3
BHB/M I.V.	4 M BHB/4.3 mM melatonin, 20% DMSO	3	3
BHB/M I.V.	4 M BHB/0.43 mM melatonin, 20% DMSO	3	3

The main focus of our analysis was to identify the effects of decreased doses of melatonin in combination with 4M BHB in pigs exposed to trauma, hemorrhage and resuscitation. To account for the decreasing samples size in the groups treated with lower doses of melatonin (due to high mortality), treatments were pooled into three groups: 1) Control (lactated Ringer's solution, n = 16), 2) high-dose melatonin (4M BHB/ 43mM Melatonin, n = 16) and 3) low-dose melatonin (4M BHB/ 0.43-20mM Melatonin, n = 22).

Parameters were analyzed using the MIXED procedure in SAS Version 9.4 software (SAS Institute, Inc., Cary, NC). Group, Time point and Group*Time point interaction were modeled as fixed effects. The models used compound symmetry, autoregressive or no covariance structure (selection based on the model with the lowest Bayesian Information Criterion value) and the between-within method for degrees of freedom. Normality in the distribution of the residuals of final models was assessed using scatter and quantile-quantile plots. For parameters with significant interaction effects, differences at individual time points were analyzed by pairwise comparisons with Tukey adjustments. Data is presented at least-squares means with 95% confidence intervals.

There was a significant difference in survival 48 hours after baseline ($p = 0.011$), with lowest mortality observed in the 4M BHB/43mM M group (1/14), followed by the LR group (5/16) and pigs treated with lower doses of melatonin (Figure 9).

Figure 9: Survival in pigs exposed to trauma and hemorrhage and treated with 4M BHB and varying doses of melatonin.

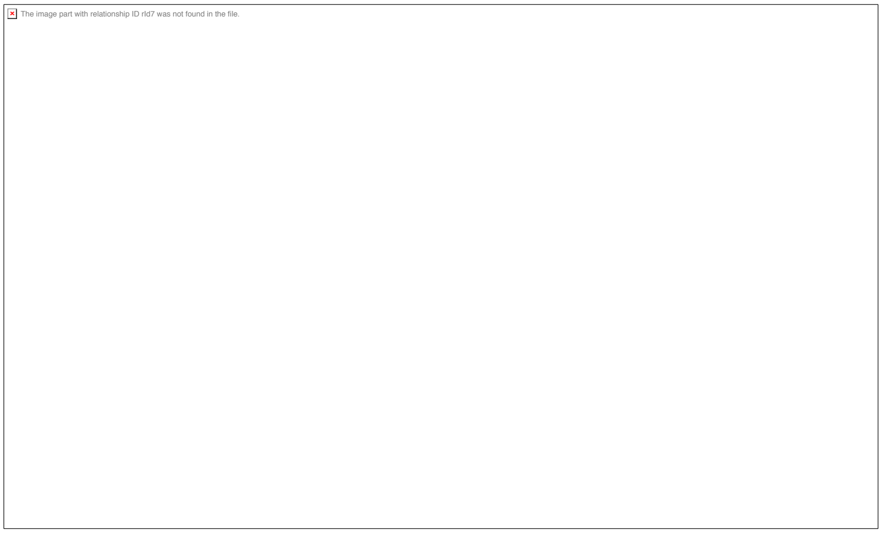

Table 7 depicts markers of organ function at key time points throughout the experiment. We identified a significant interaction effect for total protein, alkaline phosphatase and lactate dehydrogenase levels, however, review of pairwise comparisons showed no significant group differences at individual time points.

There were significant group effects for album, bilirubin, AST and creatine kinase serum levels.

Albumin levels were lowest in the low-dose melatonin group throughout the experiment. All groups showed a drop in albumin levels during the resuscitation phase, which returned to baseline levels at the end of the experiment.

Although bilirubin levels were slightly higher in the low-dose melatonin group throughout the experiment, average serum concentrations were within the normal clinical range (0.1-1.1 mg/dl) in all groups throughout the whole experiment.

Both AST and Creatine Kinase serum levels were highest in the low-dose melatonin group throughout the experiment. All groups showed increases in AST and Creatine Kinase concentrations during the resuscitation phase, which remained elevated 24 hours after the end of resuscitation.

Table 7: Markers of organ damage in pigs exposed to hemorrhagic shock and injury

Parameter	B	S45	FR2	FR20	PR 24h	Effects	
AST, U/dl							
LR	30.8 (-18.1-79.7)	111.1 (61.9-160.3)	173.3 (121.1-225.5)	99.3 (42.1-156.5)	158.0 (75.1-241)	G	0.0009
High-dose M	31.9 (-20.4-84.2)	84.0 (31.1-136.9)	196.2 (142.3-250.0)	136.2 (82.0-190.5)	152.6 (87.8-217.4)	T	<.0001
Low-dose M	41.5 (1.5-81.4)	134.0 (92.6-175.4)	293.6 (242.5-344.7)	206.3 (149.9-262.8)	212.5 (153.2-271.8)	G*T	0.3405
ALT, U/dl							
LR	63.9 (50.8-77.1)	69.4 (56.1-82.6)	72.5 (58.8-86.2)	80.9 (65.8-96.0)	135.8 (116.6-155.0)	G	0.2141
High-dose M	73.9 (59.9-88.0)	67.2 (53.1-81.4)	76.7 (62.4-91.1)	82.7 (68.1-97.3)	132.4 (116.2-148.5)	T	<.0001
Low-dose M	58.3 (47.6-69.1)	55.7 (47.6-69.1)	65.0 (52.2-77.7)	79.9 (64.8-95.1)	120.1 (104.5-135.7)	G*T	0.3943
Bilirubin, mg/dl							
LR	0.12 (-0.07-0.30)	0.14 (-0.05-0.33)	0.12 (-0.10-0.33)	0.19 (-0.03-0.41)	0.13 (-0.39-0.64)	G	0.0071
High-dose M	0.10 (-0.10-0.30)	0.12 (-0.08-0.33)	0.10 (-0.11-0.31)	0.12 (-0.09-0.32)	0.10 (-0.27-0.47)	T	0.8636
Low-dose M	0.19 (0.04-0.34)	0.20 (0.04-0.37)	0.18 (-0.03-0.40)	0.23 (0.01-0.44)	1.07 (0.83-1.32)	G*T	0.7045
Alk Phos, U/dl							
LR	226.8 (191.5-262.0)	224.0 (188.6-259.3)	224.1 (187.3-260.9)	195.4 (154.8-235.9)	227.1 (174.4-279.8)	G	0.3003
High-dose M	223.4 (185.7-261.1)	191.1 (153.1-229.0)	193.4 (154.9-231.9)	211.5 (172.4-250.5)	184.7 (141.0-228.4)	T	<.0001
Low-dose M	245.7 (216.9-274.5)	227.6 (198.3-256.8)	234.5 (200.0-269.1)	248.6 (208.0-289.2)	162.1 (120.1-204.0)	G*T	0.0051
Albumin, g/dl							
LR	2.0 (1.8-2.3)	1.9 (1.6-2.1)	1.7 (1.4-1.9)	1.6 (1.3-1.8)	2.2 (1.8-2.5)	G	0.0017
High-dose M	2.4 (2.1-2.6)	1.9 (1.7-2.2)	1.8 (1.6-2.1)	1.7 (1.5-2.0)	2.5 (2.2-2.8)	T	<.0001
Low-dose M	1.7 (1.5-1.9)	1.4 (1.2-1.6)	1.3 (1.0-1.5)	1.2 (1.0-1.4)	1.6 (1.4-1.8)	G*T	0.0762
Total Protein, g/dl							
LR	4.4 (4.2-4.6)	4.0 (3.8-4.2)	3.5 (3.3-3.8)	3.4 (3.1-5.0)	4.4 (3.9-5.0)	G	0.7119
High-dose M	4.5 (4.3-4.8)	5.6 (3.4-3.8)	3.4 (3.2-3.6)	3.5 (3.2-3.7)	5.1 (4.7-5.5)	T	<.0001
Low-dose M	4.6 (4.4-4.7)	3.8 (3.6-4.0)	3.4 (3.1-3.6)	3.6 (3.3-3.8)	4.4 (4.2-4.9)	G*T	0.039
BUN, mg/dl							
LR	13.6 (-0.4-27.7)	11.3 (-3.2-25.9)	12.8 (-3.4-29.1)	31.6 (14.6-48.5)	16.0 (-23.8-55.8)	G	0.4921
High-dose M	9.5 (-5.5-24.5)	11.9 (-3.7-27.5)	12.6 (-3.0-28.2)	14.4 (-1.2-30.0)	13.0 (-15.1-41.1)	T	0.7413
Low-dose M	9.1 (-2.4-20.6)	11.6 (-1.0-24.1)	11.8 (-4.4-28.1)	13.3 (-3.0-29.5)	77.3 (58.6-96.1)	G*T	0.7135
CK, U/dl							
LR	475 (-1506-2456)	683 (-1315-2681)	1762 (-383-3907)	3046 (706-5386)	3959 (281-7636)	G	0.0138
High-dose M	463 (-1656-2581)	626 (-1525-2777)	2090 (-99-4278)	3734 (1536-5932)	9339 (6567-12110)	T	<.0001
Low-dose M	1181 (-436-2799)	1136 (-559-2831)	3338 (1167-5508)	7481 (5193-9769)	12117 (9679-14556)	G*T	0.8144
LDH, U/dl							
LR	1122 (381-1862)	1320 (579-2061)	1882 (1132-2632)	2803 (1995-3610)	2187 (2397-4176)	G	0.701
High-dose M	1098 (307-1889)	1168 (376-1961)	2432 (1635-3230)	3067 (2254-3881)	4565 (3724-5406)	T	<.0001
Low-dose M	945 (340-1549)	1102 (494-1710)	2072 (1424-2721)	3488 (2698-4279)	3637 (2830-4443)	G*T	<.0001

Data are presented as least squared means (95% confidence interval). AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alk Phos, alkaline phosphatase; SUN, serum urea nitrogen; CK, total creatinine kinase; LDH, lactate dehydrogenase. G – group, T – Time point, G*T – group*time point interaction

Table 8 depicts different physiological parameters at key time points throughout the experiment. We did not observe significant group or interaction effects for mean pulmonary artery pressure, wedge pressure, bladder pressure, mixed venous oxygen saturation, hemoglobin, glucose, oxygen consumption, and body temperature. There were significant group and interaction effects for cardiac output, and a significant interaction effect for heart rate, however, post-hoc analysis showed no significant group differences at individual time points for either parameter.

We observed significant group effects for Mean Arterial Pressure, urine output, P:F ratio values and Calcium serum concentrations. Mean Arterial Pressure tended to be lowest in the low-dose melatonin and highest in the LR group during the resuscitation phase. Urine output was highest in the high melatonin dose group during the early and late resuscitation phase. Low-dose melatonin pigs also experienced decreased P:F ratio values which were lowest during the early resuscitation phase. Calcium serum levels were lowest in the low-dose melatonin group until the end of the resuscitation phase and highest in the LR group during early resuscitation.

We identified significant group and interaction effects ($p<0.05$) for arterial pH, base excess and serum sodium, potassium and lactate concentrations.

As previously reported by Wolf et al., arterial blood pH was lowest in the LR-treated group throughout the resuscitation phase, with a significant difference at FR2 vs. high melatonin dose (1). Accordingly, base excess was lowest in the LR group, with significant differences during the early resuscitation phase.

Interestingly, lactate serum levels were highest in the low dose melatonin group starting at the end of the shock period, with significant differences during the first two hours of resuscitation and again 24 hours after the end of resuscitation.

Na levels were significantly higher in both BHB/M groups than in the LR group throughout the first ten hours of resuscitation. Interestingly, Na levels in the low-dose melatonin group remained higher than those in the high-dose melatonin group, although these differences were not statistically significant. This may e a result of the higher DMSO concentration in the high-dose melatonin group, which is a known diuretic. As previously reported, potassium levels were higher in LR-treated pigs during the first

hours of the resuscitation phase, with no obvious differences between the low-dose and the high-dose melatonin group.

Table 8: Physiologic and hemodynamic Parameters in pigs exposed to hemorrhagic shock and injury – need to fill in values

Parameter	B	S45	FR2	FR20	PR 24h	Effects	
MPAP, mm Hg							
LR	19.9 (16.8-23.1)	16.2 (13.1-19.4)	25.6 (22.1-29.0)	18.8 (15.0-22.7)		G	0.3882
High-dose M	17.3 (13.9-20.6)	14.1 (10.6-17.5)	18.6 (15.1-22.1)	17.9 (14.4-21.5)		T	<0.0001
Low-dose M	20.3 (17.7-22.9)	14.2 (11.5-16.8)	22.3 (18.6-26.0)	21.9 (18.3-25.6)		G*T	0.6602
Wedge pressure, mm Hg							
LR	5.8 (3.8-7.8)	4.9 (2.9-6.8)	5.1 (2.9-7.2)	5.6 (3.2-8.0)		G	0.0859
High-dose M	4.5 (2.4-6.6)	3.1 (0.9-5.3)	4.6 (2.1-6.8)	5.8 (3.6-7.9)		T	0.0513
Low-dose M	4.9 (3.3-6.5)	3.8 (2.2-5.5)	3.8 (1.5-6.1)	6.1 (3.8-8.3)		G*T	0.2743
Bladder pressure, mm Hg							
LR	11.7 (9.3-14.1)	11.2 (8.8-13.5)	12.5 (10.1-15.0)	12.3 (9.6-15.0)		G	0.0776
High-dose M	9.3 (6.8-11.9)	9.4 (6.8-12.0)	10.2 (7.6-12.7)	10.2 (7.6-12.8)		T	<0.0001
Low-dose M	7.9 (6.0-9.9)	9.1 (7.1-11.1)	8.3 (6.0-10.6)	11.9 (9.7-14.2)		G*T	0.1128
SvO2, ml O2/min							
LR	72.4 (67.1-77.8)	25.9 (2.5-31.2)	65.9 (60.0-71.7)	64.1 (57.6-70.6)	56.5 (49.8-63.2)	G	0.9332
High-dose M	72.3 (66.6-78.0)	29.9 (24.1-35.8)	73.2 (67.3-79.2)	65.5 (59.5-71.4)	57.2 (51.0-63.4)	T	<0.0001
Low-dose M	75.0 (70.6-79.3)	26.2 (21.7-30.7)	62.9 (56.7-69.1)	60.1 (53.9-66.3)	61.6 (54.3-68.8)	G*T	0.1266
Hgb, g/dl							
LR	8.7 (8.4-9.0)	7.0 (6.6-7.3)	6.1 (5.8-6.5)	5.7 (5.3-6.0)	6.6 (6.3-7.0)	G	0.2888
High-dose M	8.6 (8.2-8.9)	6.1 (5.7-6.4)	6.0 (5.7-6.4)	5.7 (5.3-6.0)	6.3 (6.0-6.7)	T	<0.0001
Low-dose M	8.7 (8.4-8.9)	6.4 (6.2-6.7)	6.2 (5.8-6.5)	5.9 (5.6-6.3)	6.2 (5.8-6.7)	G*T	0.3791
Glucose							
LR	102 (88-115)	128 (115-141)	109 (94-123)	69 (52-86)	91 (74-108)	G	0.8419
High-dose M	114 (99-128)	161 (146-176)	85 (71-100)	73 (58-87)	93 (77-109)	T	<0.0001
Low-dose M	98 (87-109)	118 (106-129)	89 (73-105)	83 (67-98)	136 (113-159)	G*T	0.2426
VO2, ml O2/min							
LR	4.5 (3.9-5.2)	4.4 (3.7-5.1)	5.1 (4.4-5.8)	4.8 (4.0-5.6)		G	0.107
High-dose M	4.2 (3.5-4.9)	4.3 (3.5-5.1)	5.3 (4.5-6.1)	4.5 (3.7-5.3)		T	0.0002
Low-dose M	4.3 (3.8-4.8)	3.8 (3.3-4.4)	5.2 (4.5-6.0)	4.6 (3.8-5.3)		G*T	0.8421
Body Temperature, °C							
LR	38.0 (37.5-38.5)	38.6 (38.1-39.1)	37.9 (37.4-38.4)	39.2 (38.7-39.8)		G	0.1219
High-dose M	38.0 (37.5-38.5)	38.4 (37.8-38.9)	38.5 (38.0-39.0)	39.5 (38.9-40.0)		T	<0.0001
Low-dose M	38.4 (37.9-38.8)	39.0 (38.5-39.4)	38.9 (38.5-39.4)	39.6 (39.1-40.2)		G*T	0.1758
CO, l/min							
LR	2.9 (2.4-3.4)	1.3 (0.8-1.8)	4.4 (3.9-5.0)	4.3 (3.7-4.9)		G	0.0163
High-dose M	2.9 (2.4-3.4)	1.7 (1.1-2.3)	5.1 (4.5-5.6)	4.0 (3.5-4.6)		T	<0.0001
Low-dose M	3.4 (3.0-3.8)	1.3 (0.9-1.7)	4.4 (3.9-5.0)	3.6 (3.1-4.2)		G*T	0.034
Heart rate, beats/ min							
LR	120 (10-136)	237 (221-254)	169 (151-187)	158 (138-178)		G	0.0521
High-dose M	125 (107-143)	198 (180-216)	151 (132-169)	144 (126-162)		T	<0.0001
Low-dose M	142 (128-155)	246 (232-260)	185 (166-204)	146 (126-165)		G*T	0.0007

Data are presented as least squared means (95% confidence interval). B, baseline; S 45-min end-of-shock period; PR, post resuscitation; MPAP, mean pulmonary arterial pressure; VO2, systemic oxygen consumption; SvO2, mixed venous oxygen saturation; Hgb, hemoglobin, CO – cardiac output.

Specific Aim 3. Toxicity of BHB/M administered intraosseously (IO)

Deliverable Aim 3: Identification of NOAEL, MTD, and toxicity profile of BHB/M following IO administration and comparison with IV administration

Evaluation of the pharmacokinetics of IV and intraosseous administration of BHB/M. This is relevant especially given the finding that the intraosseous route at standard doses did not result in improved survival.

Pharmacokinetic assessment of BHB/M administered either intraosseous (IO) or intravenous (IV) was carried out using at various dosing regimens. 7 study groups with 4 animals/group (2 males and 2 females), n=28. One sacrifice time point FR 7.

A simple instrumentation procedure and drug infusion with animals randomized based on the following experimental grid (Table 9) was utilized for this aim.

Table 9.

Group	Drug Component	Concentration of Drug component	Number of animals requested
1	Lacated Ringers'	10cc/kg, 1 cc/kg bolus 0.66 cc/kg/hr	4
2	BHB/M ½ dose I.V.	4 M BHB/ 43 mM melatonin 0.5 cc/kg bolus, 0.33 cc/kg/hr	4
3	BHB/M Full dose I.V.	4 M BHB/ 43 mM melatonin 1 cc/kg bolus, 0.66 cc/kg/hr	4
4	BHB/M Double dose I.V.	4 M BHB/ 43 mM melatonin 2 cc/kg bolus, 1.32 cc/kg/hr	4
5	BHB/M ½ dose I.O.	4 M BHB/ 43 mM melatonin 0.5 cc/kg bolus, 0.33 cc/kg/hr	4
6	BHB/M Full dose I.O.	4 M BHB/ 43 mM melatonin 1 cc/kg bolus, 0.66 cc/kg/hr	4
7	BHB/M Double dose I.O.	4 M BHB/ 43 mM melatonin 2 cc/kg bolus, 1.32 cc/kg/hr	4

I.V.= intravenous, I.O.=intraosseous

Melatonin and β-hydroxybutyrate (BHB) concentrations were obtained from the above outlined pharmacology experiments. Although the difference is not statistically significant, our results suggest that both melatonin and BHB concentrations are lower in animals receiving an intraosseous, full-dose infusion of BHB/M (green diamonds) when compared to the animals receiving an intravenous, full-dose infusion of BHB/M (blue triangles) (Figure 10 and 11). Interestingly, serum levels of both melatonin and beta-hyroxybutyrate in animals receiving BHB/M intraosseously, at full doses were similar to levels of animals receiving intravenous, half-dose.

We conclude that intraosseous infusion of BHB/M is associated with lower serum levels of both components at the currently utilized dose. The reason for this is unclear, but may be related to either bone marrow clearance or absorption, especially of the BHB component of the treatment. These decreased levels are likely below levels that we believe are associated with therapeutic effect (6-8mM).

Figure 10. Melatonin concentrations in animals receiving intraosseous and I.V. infusion of BHB/Melatonin at three doses compared to animals receiving lactated Ringers'. Animals were instrumented but did not undergo injury or shock protocol.

Figure 11. BHB concentrations in animals receiving intraosseous and I.V. infusion of BHB/Melatonin at three doses compared to animals receiving lactated Ringers'. Animals were instrumented but did not undergo injury or shock protocol.

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Throughout the pharmacology arm of this study, we did not find any significant physiologic changes that occurred in animals receiving half dose, full dose, or double dose BHB/M. There were no deaths and no adverse events.

There were identifiable increases in pH in animals receiving BHB/M, with the highest pH reached of 7.635. This trend reached significance at two hours after infusion was started, and stayed significant throughout the infusion period for high doses. This effect resolved quickly after completion of infusion (Figure 12). We have previously described this effect (2). The cause is unclear. In a human experiment of infusion of BHB compared to glucose, BHB infusion was associated with an increase in bicarbonate production and a decrease in respiratory exchange ration (3). It is unclear whether this is an effect of the BHB on cellular metabolism, a chemical effect of the treatment, or an effect of this organic acid and renal tubular cells.

Figure 12. pH in animals receiving varied dose of BHB/M compared to animals receiving infusion of lactated Ringers'.

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There were notable trends and differences in sodium concentrations in serum during infusion of double-dose BHB. At baseline, there were no significant differences in sodium levels between groups. Over the course of the experiment, there was a trend toward hypernatremia in the treatment groups, with the sodium levels in double-dose groups remaining above 150 mM/L one hour after bolus and infusion. The significant increase in sodium continued in the double-dose treated animals throughout the experiment, with double-dose groups having significantly higher sodium levels than the control group, as well as the half-dose I.O. group (Figure 13A).

There were also notable trends and differences in serum potassium levels during infusion. At baseline, there was no significant difference between groups. There is a noticeable trend during the initial post-infusion time points, for hypokalemia in the treatment groups. Beginning at one hour after bolus and infusion, there were significantly lower potassium levels in most experimental groups when compared to lactated Ringers'-infused, controls. This trend continues throughout infusion, with serum

potassium returning towards baseline with interruption of infusion in all groups (Figure 13B).

Figure 13. Serum sodium and potassium levels in animals receiving varied dose of BHB/M compared to animals receiving infusion of lactated Ringers'. A) Serum sodium levels and B) serum potassium levels.

A)

B)

We conclude that the sodium and potassium changes are dose-related and trasient after administratuion of BHB/M. These changes were not associated with abnormalities in renal or cardiac function.

Of note, there were no significant differences between the groups found in the heart rate, cardiac output, oxygen requirements or delivery, blood pressure, or urine output. There was also no significant difference between groups in liver function tests, no change in BUN and no change in creatinine kinase. There was no laboratory evidence of damage or toxic effect to the function of major organs. Although there were notable swings in blood glucose levels, with intermittent difference being significant, there was no correlation of hypo- or hyperglycemia with changes in the drug dosage.

At the end of the experiments (~4.5 hours after the completion of infusion), necropsies were performed. The following tissue/organs were grossly inspected; adrenal glands, bones, brain, esophagus, eyes gall bladder, heart and great vessels, intestine both large and small, both left and right kidneys, liver, lymph nodes, left and right lung, oral cavity and tongue, pancreas, reproductive tract, skin, sternum bone marrow, stomach, thymus, thyroid, trachea and bronchi, urinary bladder and vessels. Histology was read from the following; heart, rib tibia, right and left kidney, right and left lateral liver lobe, thyroid gland, small intestine, large intestine, stomach gall bladder, adrenal glands, thymus pancreas, mesenteric lymph node, submandibular lymph node, urinary bladder, right and left lung, carotid artery, pituitary gland, cervical spinal cord, thoracic spinal cord, lumbar spinal cord, and brain. All necropsies and histology were performed/read either by George Ruth DVM, PhD, DACVP or Nick Robinson, BVSc, PhD, MACVSc, DACVP, Veterinary Pathologists contracted by Experimental Surgical Services. Pathology review in these animals confirmed the findings at our previous studies, with no significant abnormalities noted in BHB/M infused animals when compared with controls.

Pathology for this task indicates that there are no detrimental effects when BHB/M is administered either intra-venous or intra-osseous in various doses.

Key Research Accomplishments:

* Completed rabbit ear vein studies. 4M BHB/M can be administered I.V. if given at pH 7.4 .
* Completed our assessment of BHB/M administered either intraosseously (IO) or intravenously (IV).
* Completed assessment of BHB/M administered either intraosseous (IO) or intravenous (IV) at various dosing regimens.
* Confirmed survival benefit of treatment with 4M BHB/43mM Melatonin in 20% DMSO in model of shock simulating battlefield injury.

Conclusion:

- A pH-neutral mixture of 4M-d-beta Hydroxybutyrate and 43mM-Melatonin solution is not associated with long-term severe vascular or tissue necrosis and is safe to administer via peripheral vein.
- BHB/M at full dose given intraosseously did not result in improved survival compared with controls. This lack of effect appears to be related to blood levels of BHB (and potentially melatonin) given I.O. rather than I.V.
- BHB/M appears to be safe, when given both I.V. and I.O., with no identified histopathologic changes or physiologic changes associated with dose tested.
- Melatonin given at 43mM, 4.3mM or 0.43mM in conjunction with 4M BHB was used to treat animals undergoing shock, poly-trauma and resuscitation. Injured animals receiving 4M BHB/43mM melatonin demonstrated a survival benefit when compared to animals receiving 4M BHB/4.3mM Melatonin, 4M BHB/0.43mM Melatonin or L.R.
- Based on the date presented, while animals treated with 2X 4 M BHB/43mM Melatonin did have death as well as other physiologic and hematologic changes associated with experimental outcome, animals treated with 4X 4 M BHB/43mM Melatonin had more severe outcomes and measures. Therefore, improved outcomes are not associated with a higher dose of BHB/M and in fact would appear detrimental.
- The individual components given I.V. to the injured animals at MTD (4X, determined in the previous set of experiments) resulted in death in 7/9 animals. Non-injured animals that received MTD (4X) of the individual components survived until the end of the experiments (8/8). It is concluded that it is likely that the animals died from a combination of both the injury and the high dosing of drug.

Publications, Abstracts, and Presentations:

* Rabbit ear vein studies were presented at ATACCC in the Advanced Technology Applications for Combat Casualty Care in August 15-18, 2011 in Fort Lauderdale, FL.

* Wolf A, Mulier KE, Iyegha UP, Asghar JI, Beilman GJ. Safety of D-β-Hydroxybutyrate and Melatonin for the Treatment of Hemorrhagic Shock with Polytrauma. Shock 2015 1:79-89

* Poster presentation at the Doctoral Research Showcase Minneapolis, MN. April 2016. D-B-hydroxybutyrate and Melatonin as a novel Treatment for Hemorrhagic Shock

* Poster presentation at the 39th Annual Conference on Shock, Austin, Texas, June 2016. Wolf A, Mulier KE, Muratore SL, Beilman GJ, Efficacy to Improve Survival of Combinations of 4M D-beta-Hydroxybutyrate and Varying Melatonin Concentrations in a Porcine Hemorrhagic Shock Model.

References:

1. Wolf A, Mulier KE, Iyegha UP, Asghar JI, Beilman GJ. Safety of D-β-Hydroxybutyrate and Melatonin for the Treatment of Hemorrhagic Shock with Polytrauma. *Shock*. 2015 1:79-89.

2. Mulier KE, Lexcen DR, Luzcek E, Greenberg JJ, Beilman GJ. Treatment with beta-hydroxybutyrate and melatonin is associated with improved survival in a porcine model of hemorrhagic shock. *Resuscitation*. 2012 83(2):253-8.

3. Chiolero R, Mavrocardatos P, Burnier P, Cayeux MC, Schindler C, Jeguier E, Tappy L. Effects of infused sodium acetate, sodium lactate, and sodium beta-hydroxybutyrate on energy expenditure and substrate oxidation rates in lean humans. *Am J Clin Nut*r. 1993 58(5):608-13.

Appendices:
Timepoints defined, Limited Resuscitation (LR)=maintenance of SBP above 80 mmHg, Full Resuscitation (FR)=maintenance of SBP above 90 mmHg, Hgb above 6 and Urine output > 1 cc/kg/hr.

Timepoint	**Elapsed time from Baseline**
Baseline	0
Shock 15	15 minutes
Shock 35	35 minutes
Shock 45	45 minutes
LR 30	30 minutes from the start of Limited Resuscitation phase, ~1.5 hours from baseline
LR 1	60 minutes from the start of Limited Resuscitation phase, ~2 hours from baseline
FR 1	1 hour from the start of Full Resuscitation, 2 hours from the start of Limited Resuscitation, ~3 hours from Baseline
FR 2	2 hour from the start of Full Resuscitation, 3 hours from the start of Limited Resuscitation, ~4 hours from Baseline
FR 160	160 minutes from the start of Full Resuscitation, 3 hours 40 minutes from the start of Limited Resuscitation, ~4.7 hours from Baseline
FR 170	170 minutes from the start of Full Resuscitation, 3 hours 50 minutes from the start of Limited Resuscitation, ~4.83 hours from Baseline
FR 3	3 hour from the start of Full Resuscitation, 4 hours from the start of Limited Resuscitation, ~5 hours from Baseline
FR 190	190 minutes from the start of Full Resuscitation, 4 hours 10 minutes from the start of Limited Resuscitation, ~5.2 hours from Baseline
FR 4	4 hour from the start of Full Resuscitation, 5 hours from the start of Limited Resuscitation, ~6 hours from Baseline
FR 5	5 hour from the start of Full Resuscitation, 6 hours from the start of Limited Resuscitation, ~7 hours from Baseline
FR 6	6 hour from the start of Full Resuscitation, 7 hours from the start of Limited Resuscitation, ~8 hours from Baseline
FR 7	7 hour from the start of Full Resuscitation, 8 hours from the start of Limited Resuscitation, ~9 hours from Baseline
FR 20	20 hour from the start of Full Resuscitation, 21 hours from the start of Limited Resuscitation, ~22 hours from Baseline